

Antigen-specific targeting and elimination of EBV-transformed B cells by allergen toxins

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Background: With the exception of antigen-specific immunotherapy, current treatments for atopic diseases provide only symptomatic relief. Because of the increasing incidence of such diseases, the development of novel strategies and concepts for the treatment of allergies is urgently needed. **Objective:** Here we present a new approach for the treatment of atopic diseases. The strategy is comparable to the application of immunotoxins in cancer therapy, in which a cytotoxic peptide is coupled to a cancer cell-specific antibody fragment or ligand. In the case of so-called *allergen toxins* (ATs), the target cell-specific moiety is an allergen or allergen-derived fragment, which should be bound only by allergen-reactive cells. After receptor-mediated internalization, allergen-specific cells are killed, and the allergic pathogenesis is interrupted. **Methods:** Proof of the AT principle was shown by using a human *ex vivo* system in which EBV was used to transform human B cells specific for the timothy grass pollen allergen Phl p 5b. The AT is composed of the major B-cell and T-cell epitopes of the Phl p 5b (P5) allergen fused to a truncated form of the highly toxic *Pseudomonas aeruginosa* exotoxin A (ETA'). **Results:** Allergen-specific and nonspecific B cells were challenged with P5-ETA', but only the Phl p 5b-reactive B cells showed selective binding and cytotoxicity. **Conclusion:** This approach represents an initial step toward a novel therapeutic strategy in the treatment of atopic diseases. (J Allergy Clin Immunol 2005;116:910-5.)

Key words: Antigen, allergen, EBV transformation, B-cell targeting, allergen toxin

Allergic diseases now affect as much as 20% of the world's population, and this is becoming an increasing

Abbreviations used

AT: Allergen toxin
BCR: B-cell receptor
ETA': Catalytic domain of *Pseudomonas aeruginosa* exotoxin A
FACS: Fluorescence-activated cell sorting
MACS: Magnetic adsorbent cell sorting
P5: Recombinantly truncated form of Phl p 5b

burden to the national health systems particularly of industrialized countries. Improved understanding of the pathophysiology of atopic diseases has led to the development of novel treatment strategies. These include the application of phosphodiesterase inhibitors,¹ the development of cytokine modulation strategies,^{2,3} and treatments based on anti-IgE.⁴ Despite the success of these treatments, they address the symptoms of the disease rather than the cause and hence require long-term application. Until now, the only approach that can cure atopic diseases has been antigen-specific immunotherapy, which involves the administration of recombinant allergen or allergen extracts.⁵⁻⁷ However, specific immunotherapy can have side effects, including in some cases lethal anaphylactic shock, and this has limited its clinical use.⁸

Another potential curative approach is the targeted elimination of allergen-reactive cells by the fusion of allergens with a cytotoxin. This strategy is equivalent to the use of immunotoxins for the treatment of cancer, an approach in which a target cell-specific binding domain is fused to a cytotoxic compound.⁹ Although exclusive targets can be quite difficult to find in cancer therapy, the design of target cell-specific allergen toxins (AT) is more straightforward. Allergen-reactive cells are defined by the presence of allergen-specific immunoglobulins, such as B-cell receptors (BCRs) or Fc-receptor-bound antibodies. Therefore, a cytotoxin fused to an allergen or allergen fragment should also bind specifically to these cells. Subsequent receptor-mediated internalization of the AT results in cell death mediated by the cytotoxic domain. Thus, the target cell populations will be composed of antigen-presenting cells, including allergen-specific B cells. Several similar approaches to eliminate antigen-specific cells have been investigated, including the

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treatment of autoreactive immune disorders¹⁰⁻¹² as well as models for atopic diseases.¹³ Recently, the protection of ovalbumin-sensitized mice from systemic anaphylactic shock by the administration of a recombinant ovalbumin toxin has been reported.¹⁴ Further investigations correlated this finding with a treatment-induced immunoglobulin class switch from ovalbumin-specific IgE to IgG.¹⁵

One major drawback of these experiments is their limitation to an artificial allergen/mouse system. Future advances may benefit from the use of more clinically relevant allergens and humanized models. We have therefore focused on the establishment of a human *ex vivo* model as a basis for further investigations.

As stated, the AT is composed of 2 functionally distinct domains. We have chosen the Phl p 5b allergen for targeting and binding to allergen-specific cells because of its clinical relevance. The protein has been classified as a major allergen because at least 80% of timothy grass pollen-reactive individuals produce Phl p 5b-specific IgE. Furthermore, the protein has been well characterized and expressed in bacteria.¹⁶ P5, the first 205 amino acids comprising the major B-cell and T-cell epitopes of the Phl p 5b allergen,¹⁷ was fused to a modified *Pseudomonas aeruginosa* exotoxin A (ETA') lacking the natural cell binding domain¹⁸ to obtain the final AT. ETA' is highly toxic and has been shown to function efficiently in different immunotoxins.^{19,20}

Because of their scarcity, sufficient numbers of antigen-specific cells cannot be prepared directly from peripheral blood. EBV-transformed B cells are known for their strong antigen presentation activity^{21,22} and dispose of BCRs.²³ We first selected allergen-specific B cells by high gradient magnetic adsorbent cell sorting (MACS).²⁴ The subsequent EBV transformation then allowed us to cultivate allergen-specific and nonspecific B-cell clones.

METHODS

Bacterial strains, Phl p 5b allergen, oligonucleotides, and cloning

Escherichia coli strain XL1-Blue (*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lacF'* [*pro AB*⁺ *lacI*^q *lacZ*ΔM15 Tn10(*tet*^r)]) was used for plasmid propagation. *E. coli* strain BL21 Star (DE3; Stratagene, La Jolla, Calif) (F[−] *ompT hsdS_B(r_B[−]m_B[−]) gal dem rne131* DE3) was used for the synthesis of recombinant immunotoxins. Synthetic oligonucleotides were synthesized by MWG Biotech (Ebersberg, Germany). The bacterial expression vector pBM1.1, which is derived from the pET27b plasmid (Novagen, Madison, Wis), was used for 5'-end fusion of *Sfi* I/*Not* I-binding structures to the modified deletion mutant of *P. aeruginosa* exotoxin A.¹⁸ The Phl p 5b sequence (GenBank accession number AF069473) was originally obtained from a timothy grass pollen cDNA library.¹⁶ All standard cloning procedures were performed as described by Sambrook et al.²⁵ Recombinant Phl p 5b¹⁶ was biotinylated by using the EZ-Link Kit (Pierce, Rockford, Ill) according to the manufacturer's instructions.

Plasmid cloning and bacterial expression of P5-ETA'

To clone the timothy grass pollen allergen Phl p 5b cDNA as part of an ETA'-based AT, it was necessary to introduce a 5' *Sfi* I

restriction site by PCR (pp5b-back primer: AAG CTT TTA TGG CCC AGC CGG CCG ccg atg ccg gct acg gc; bold letters: *Sfi* I; pp5b-for: gct gcc ggt gcc tat aaa gtc). The 3' *Not* I restriction site was already part of the Phl p 5b cDNA and was located at amino acid position 205. This site is in frame with the target *Not* I site in pBM1.1. Because the most relevant B-cell epitopes were located upstream of this site,¹⁷ we used this *Not* I site for the final cloning step. PCR amplification was performed by using a Primus thermocycler (MWG, Martinsried, Germany) and the following cycling conditions: 96°C, 5 minutes; 30× (96°C, 1 minute; 60°C, 1 minute; 72°C, 1 minute); 72°C, 4 minutes. This produced the expected 842-bp PCR fragment. After subsequent digestion with *Sfi* I and *Not* I, the resulting 627-bp P5 restriction fragment was inserted into the bacterial expression vector pBM1.1, which had been digested with the same restriction enzymes. The final structure of the P5-ETA' expression cassette is shown in Fig 1. To verify cloning, the plasmid was sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM sequencer (Applied Biosystems, Foster City, Calif). As a negative control for the different functional assays on EBV-transformed B cells, we used the previously described anti-epidermal growth factor receptor immunotoxin 425(scFv)-ETA'.²⁶ Expression of recombinant P5-ETA' and 425(scFv)-ETA' was achieved after the introduction of pBM-P5-ETA' and 425(scFv)-ETA' into BL21 Star (DE3). The ETA' fusion proteins were expressed in the periplasm under osmotic stress in the presence of compatible solutes⁴, purified, and characterized as described by Barth et al.²⁷ Briefly, after isopropyl-beta-D-thiogalactopyranoside (IPTG)-induced expression from 1 L bacterial culture, 20 g bacterial pellet was harvested. Final yield of P5-ETA' after Ni-NTA (Qiagen, Hilden, Germany) chromatography was 1.2 mg with a purity of 90%. Subsequent desalting and size exclusion chromatography increased the purity to >95%.

ELISA

ELISAs were performed on 96-well microtiter Maxisorb plates (Nunc, Wiesbaden, Germany). Plates were coated with recombinant bacterial P5-fusions as described.²⁸ Bound immunoglobulins were detected with appropriate alkaline phosphatase-conjugated antibodies. The different antibodies used for detection of isotypes were goat antihuman IgM and goat antihuman IgE (Kirkegaard, Gaithersburg, Md), rabbit antihuman IgA, rabbit antihuman IgG₁, and rabbit antihuman IgG₄ (Sigma, Deisenhofen, Germany). Negative values resulted from background adjustment and averaged controls. For visualization, the plates were incubated with a colorimetric substrate solution ([2,2'-azino-diethylbenzthiazolinsulfonate] ATBS; Boehringer Mannheim, Mannheim, Germany); the resulting absorption was measured at 495 nm.

SDS-PAGE and Western blotting

SDS-PAGE, Coomassie staining, and Western blotting were performed as previously described.²⁹ Recombinant ETA'-constructs were detected by using a mouse anti-penta-His mAb (Qiagen). Bound antibody was visualized by using a horseradish peroxidase-conjugated donkey antimouse-IgG mAb (Dianova, Hamburg, Germany) followed by enhanced chemi-luminescence (Pierce) mediated chemi-luminescence detection. The light emission was photographed with a LAS-1000 luminescence image analyzer (Fujifilm, Tokyo, Japan).

Patient material and control sera

On the basis of UniCAP (Pharmacia, Uppsala, Sweden) analysis, 3 individual sera were selected according to specific IgE levels against timothy grass pollen (CAP classes 3 and 6). CAP class 0 tested sera were used as negative controls. For the generation of immortalized,

^aPCT-Application: WO 00/28050

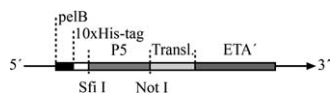


FIG 1. P5-ETA' expression cassette in pBM 1.1 (pBM-P5-ETA'). Translation starts with a *pelB*-leader sequence, which mediates the cotranslational transport of the protein into the periplasmic space. This leader peptide is followed by a 10-fold histidine tag, which facilitates purification of the AT by affinity chromatography. The P5-fragment (AA 1-205 of Phl p 5b) is fused to the translocation (*Transl.*) and the catalytic domain (ETA') of *P aeruginosa* exotoxin A.

EBV-transformed human B cells, 40 mL blood was taken from a 30-year-old male volunteer with a proven history of grass pollen allergy.

Cell culture

In general, all cells were cultivated in complex medium (RPMI 1640) supplemented with 10% (vol/vol) heat-inactivated FCS, 50 μ g/mL penicillin, 100 μ g/mL streptomycin, and 2 mmol/L L-glutamine. All cells were cultured at 37°C and 5% CO₂ in a humidified atmosphere. PBMCs were obtained from human red blood by using a Ficoll Histopaque density gradient.³⁰

Antigen specific B-cell sorting by MACS

Antigen specific B-cell sorting was achieved by using 2 consecutive high gradient-MACS sorting steps²⁴ with the OctoMACS system and the CD 19 MultiSort Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. As a source for Phl p 5b specific B cells, we used PBMCs from a Phl p 5b-reactive patient.

Paramagnetic anti-CD 19 beads were used to separate B cells from PBMCs. After enzymatic release of the beads, cells were labeled with biotinylated Phl p 5b and fractionated via streptavidin-coated microbeads. The allergen-reactive B cells were subsequently EBV-transformed (see *Methods* in the Online Repository in the online version of this article at www.jacionline.org).

EBV transformation and generation of Phl-p5-specific B cells

The allergen-specific CD19⁺ cell fraction was transferred to the CD19[−] selected PBMC fraction. The cell density was adjusted to 1 $\times$ 10⁶ cells/mL with RPMI 1640, 10% FCS, 4 μ g cyclosporin A/mL. The sample was mixed at a 4:1 (vol/vol) ratio with EBV-containing supernatant (B95.8 marmoset cell line³¹) and seeded out on 96-well microtiter plates at 100 μ L/well. After 2 to 3 weeks without further feeding, B-cell clusters were collected and propagated in 6-well plates and subsequently in 175-cm² cell culture flasks for long-term cultivation.

Flow-cytometric analyses

The AT-binding activity of EBV-transformed B cells was evaluated by flow cytometry as described previously.³² The final detection of bound proteins was made by Alexa488-conjugated anti-penta-His antibody (Qiagen, Hilden, Germany) and analyzed on a FACSCalibur (Becton Dickinson, Heidelberg, Germany).

Colorimetric cell proliferation assay

The cytotoxic effect of P5-ETA' and the cytotoxic selection marker Zeocin (Invitrogen, Carlsbad, Calif) on EBV-transformed target cells was determined by measuring the conversion of yellow 23-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT sodium salt; Sigma-Aldrich, St Louis, Mo) to the water-soluble orange formazan dye as previously described²⁹ (see *Methods* in the Online Repository in the online version of this article at www.jacionline.org). The reduction in protein synthesis relative to

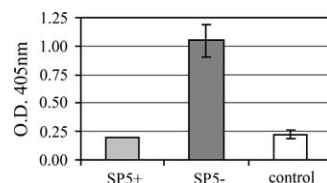


FIG 2. Binding of antibodies secreted by EBV-transformed B cells to native recombinant Phl p 5b. Recombinant Phl p 5b protein was incubated with supernatants of Phl p 5b negative selected (SP5[−]) and positive selected (SP5⁺) EBV-transformed B cells. The negative control was performed with PBS instead of B-cell supernatant.

untreated control cultures and to Zeocin-treated positive controls was calculated graphically by using Microsoft Excel. The rate of Zeocin-mediated cell killing was additionally confirmed by counting trypan blue-stained cells. All measurements were performed in triplicate (n = 3), and the SD was plotted.

RESULTS

Establishment of Phl p 5b-specific and EBV-immortalized B-cell clones

One of the major obstacles preventing the investigation of allergen toxins and their effect on human allergen-specific B cells *in vitro* is their scarceness. The establishment of allergen-specific and EBV-immortalized B cells has solved this problem. The selection of Phl p 5b-specific B cells started with $3.4 \pm 0.7 \times 10^7$ PBMCs obtained from whole blood by using a Ficoll Histopaque density gradient. Among these leukocytes, $10.6 \pm 0.6\%$ were B cells that could be isolated by the first CD19-specific selection. Through the second antigen-specific MACS procedure, 0.01% to 0.05% of this CD19⁺ cell fraction was recovered. These apparently allergen-specific B cells were then immortalized by EBV transformation for long-term cultivation.

Initial analysis 3 weeks after transformation focused on the general characterization of antibodies secreted by these B cells. Most of the antibodies detected in the cell culture supernatants were of the IgG₁ and IgM classes (data not shown).

B cells were isolated by several rounds of MACS to prevent them being overgrown by nonspecific cells. This strategy led to the establishment of both Phl p 5b-specific and Phl p 5b-nonspecific B-cell clones. In different experiments, the supernatants of different subcloned B-cell populations were tested for the existence of Phl p 5b-specific antibodies as well as for their immunoglobulin subclasses. Only IgM antibodies bound to recombinant Phl p 5b. This was shown by using denatured Phl p 5b in Western blot experiments (data not shown) as well as native Phl p 5b in ELISA experiments (Fig 2).

Expression and characterization of P5-ETA' AT

After successful expression and purification (Fig 3), initial characterization of P5-ETA' focused on the structural properties of the allergen. Different sera from timothy grass pollen-sensitive and nonallergic patients were tested

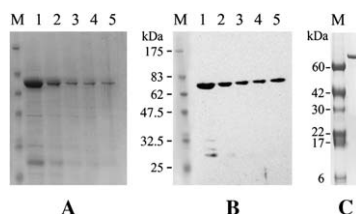


FIG 3. Coomassie-stained SDS-PAGE gels and Western blot of purified P5-ETA'. **A and B,** Five consecutive fractions of the Ni-NTA eluted P5-ETA' were analyzed. **A** Coomassie-stained SDS-PAGE gel (**A**) is shown in comparison with Western blotted and immunohistochemically detected P5-ETA' (**B**). **C,** The fractions 1-5 were pooled, desalted, purified by size exclusion chromatography, and visualized on a Coomassie-stained SDS-PAGE gel.

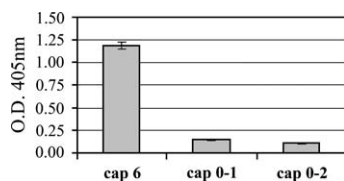


FIG 4. Binding of sera-derived IgE antibodies to P5-ETA'. Recombinant P5-ETA' was coated onto Maxisorp plates (Nunc) and incubated with sera from 1 Phl p 5b⁺ (CAP 6) and 2 Phl p 5b⁻ (CAP 0-1/2) patients.

for their ability to bind P5-ETA'. As anticipated, only IgE from the sera of atopic patients were able to bind the AT on Western blots (data not shown). Additional ELISA experiments confirmed these results. Moreover, the outcome of this experiment with native AT indicates that the recombinant bacterial AT has the correct structure (Fig 4).

Specific binding of P5-ETA' allergen toxin to Phl p 5b-reactive B cells

To confirm the value of allergen-specific EBV-transformed B cells as an *in vitro* tool for the analysis of AT, we needed to demonstrate the presence of B-cell receptors that bound to the AT and the selected Phl p 5b-specific B cells. With this in mind, 2 different types of fluorescence-activated cell sorting (FACS) analysis were performed (Fig 5). First, we showed that binding of P5-ETA' is not dependent on the cytotoxic domains in the AT. This was confirmed by the use of a B-cell-irrelevant EGF-receptor-specific 425(scFv)-ETA' immunotoxin, which differs from P5-ETA' only in the target cell-binding domain. As expected, the 425(scFv)-ETA' did not bind to Phl p 5b-specific B cells (Fig 5, A). To exclude the possibility of Phl p 5b-mediated cross-reactivity to different B cells, Phl p 5b-specific and Phl p 5b-unspecific B cells were compared for their ability to bind P5-ETA'. A signal was obtained only with the Phl p 5b-selected B cells (Fig 5, B).

Allergen-specific elimination of EBV-transformed B cells

Initial cytotoxicity assays performed with recombinant Phl p 5b on allergen-specific B cells did not show any toxic effect (data not shown). Having documented the target cell-specific binding of P5-ETA', XTT viability assays were performed. Phl p 5b-specific and Phl p 5b-unspe-

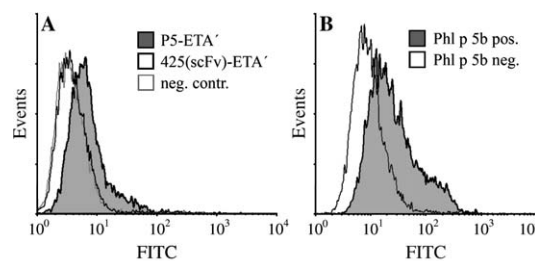


FIG 5. P5-ETA'-specific FACS analysis. **A,** The Phl p 5b-selected EBV-transformed B cells were incubated either with P5-ETA' or with the EGF receptor-specific scFv425-ETA'. In a further negative control, cells were treated with a blank reagent lacking recombinant protein. **B,** Phl p 5b⁺ versus Phl p 5b⁻ EBV-transformed B cells were incubated with P5-ETA'. FITC, Fluorescein isothiocyanate.

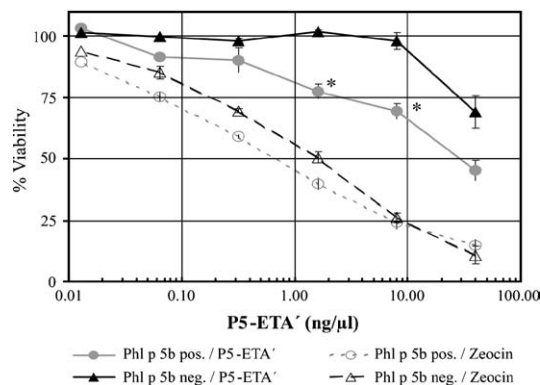


FIG 6. XTT viability assay with P5-ETA' on Phl p 5b⁺ and Phl p 5b⁻ selected EBV-transformed B cells. Phl p 5b⁺ and Phl p 5b⁻ selected EBV-transformed B cells were incubated either with increasing amounts of purified P5-ETA' or with Zeocin. Measurements were performed in triplicate (n = 3); error bars indicate SD. *P < .03 compared with the Phl p 5b⁻ selected B cells.

cific B cells were challenged with serial 1-in-5 dilutions of the purified P5-ETA' AT (Fig 6). The effect on Phl p 5b-specific B cells increased in a dose-dependent manner. In comparison, the Phl p 5b-unspecific cells did not show any effect when incubated with the AT in concentrations as high as 10 ng/μL.

DISCUSSION

In this article, we discuss for the first time a human *ex vivo* system which can be used to evaluate the allergen-specific targeting and elimination of B cells. The major findings of our work are the following: (1) EBV-transformed and immortalized human B cells meet all requirements as a system to study antigen-specific effects *in vitro*; (2) the recombinant bacterial fusion of Phl p 5b and the cytotoxic *P aeruginosa* exotoxin A combines the native structural and functional properties of both fusion partners; and (3) allergen-specific B cells can be eliminated exclusively by fusing their target antigen with a cytotoxic component.

EBV-transformed human B cells have been used for many different purposes.³³⁻³⁵ However, only 1 previous

study has described the use of this system for the propagation of allergen-specific cells.²⁴ Even though we also started with primary cells from an atopic patient, we were not able to establish IgE-secreting B cells. In an early phase of the allergen-selective cultivation of EBV-transformed B cells, IgM and IgG were detected in the supernatant. With ongoing selection and subculture of the antigen-specific B cells, the loss of IgG was observed. We assume that the initial detection of IgG reflected the presence of residual untransformed IgG-secreting B cells. These cells were lost through progressive subcultures until finally only IgM-secreting B cells remained. This observation fits the previously described preferential EBV-immortalization of IgM-secreting B cells.³⁶⁻³⁸ In 1 case, the detection of IgE-secreting B cells after EBV transformation was attributed to a class switch induced by the culture conditions.³⁹

After sorting and establishment of allergen-specific and nonspecific B-cell populations, the supernatants were tested for allergen specificity. Whereas no allergen-binding antibodies were detected in the negative selected B-cell supernatant, the positive selected B cells secreted allergen-binding IgM antibodies. In ELISA and Western blot experiments, these secreted antibodies showed no difference in their binding activity to the recombinant Phl p 5b allergen. With the successful immortalization and cultivation of allergen-specific and nonspecific B cells, we have established the basis for the further experiments.

The successfully cloned and expressed Phl p 5b-ETA' AT displays several features distinct from the Phl p 5b used to sort the allergen-specific cells. As a result of the cloning strategy, the allergen component in the AT lacks 60 C-terminal amino acids. The fusion of the truncated ETA to the C-terminus also may influence the structure and accessibility of certain epitopes for antibody binding. Furthermore, the Phl p 5b used to sort the allergen-reactive cells was expressed by standard methods,¹⁶ whereas the ETA'-based AT was generated by periplasmic expression under stress conditions.²⁷ Different expression conditions may also influence the folding of the expressed proteins. Therefore, we needed to prove that the P5-ETA' AT was still recognized by Phl p 5b-specific antibodies. The exclusive binding to P5-ETA' of IgE antibodies isolated from the sera of Phl p 5b-reactive patients indicates that the epitope remains in the correct structural confirmation within the fusion protein. The AT's target specificity for Phl p 5b-reactive EBV-transformed B cells is a prerequisite for successful XTT viability assays. The specific binding of P5-ETA' to Phl p 5b-specific B cells was demonstrated in additional FACS experiments.

Although the cytotoxic effect of the AT on EBV-transformed B cells was not as potent as previously demonstrated with cancer-related immunotoxins,^{20,40} the effect was still target cell-specific. Moreover, our results support data obtained in comparable models, in which hybridoma cells have been exposed to their target antigens fused to different toxic components.¹¹ There may be several explanations for the observed 100-fold to 1000-fold reduced AT cytotoxicity compared with classic immunotoxin. First, different cell types may vary in their sensitivity

to ETA'-based fusion proteins. This is dependent predominantly on receptor availability and the efficiency of internalization.^{41,42} The relatively small shift in our FACS analysis implies either relatively low antigen binding or low receptor density on the cell surface. Second, in the case of B cells or hybridoma cells, the secreted AT-specific antibodies compete with cell membrane bound antibodies for binding sites. Third, the expression of the EBV gene latent membrane protein 1 (*LMP-1*) leads to an impaired B-cell receptor uptake.^{21,43} Therefore, the internalization of BCRs bound to AT could be limited by the constitutive membrane turnover rate rather than by an induced receptor-mediated internalization processes. We anticipate that allergen toxins will eliminate primary allergen-reactive B cells capable of BCR internalization more efficiently.

Further predictions concerning the AT targets and their effects *in vivo* are speculative. Depletion of allergen specific B-cell populations might affect the inflammatory response at least in 2 ways. First, elimination of newly upcoming allergen-specific naive B cells will finally reduce the number of corresponding IgE-producing B cells. As a consequence, repeated applications of the AT may be necessary. Second, the loss of antigen-presenting B cells may have an influence the development of corresponding CD4⁺ memory cell populations. Because of the importance of T-cell/B-cell interactions for maintenance of atopic processes, a T-cell-directed AT strategy might be required. Taking into account the unique T-cell receptor-allergen binding characteristics, T-cell-specific AT might be feasible.^{17,44}

Evidence that the AT system has potential therapeutic value has been provided by Lee et al.^{14,15} They fused ovalbumin to the cytotoxic domain of diphtheria toxin and introduced the fusion protein into ovalbumin-sensitized mice, preventing anaphylactic reactions induced by ovalbumin alone. Further experiments revealed that this reflected a shift from the IgE to the IgG response. Interestingly, this effect can also be achieved when a nontoxic but highly immunogenic protein is fused to an allergen.⁴⁵ Therefore, it remains to be established whether the observed *in vivo* effects of allergen toxins are dependent mainly on immune-modulatory effects or on the elimination of allergen-reactive cells. The direct elimination of IgE-secreting B cells *in vivo* has not been documented thus far.

CONCLUSION

We conclude that our data, obtained by using the human *ex vivo* system described in this article, provide the basis for further studies focusing on primary human allergen-reactive B cells. Our ongoing investigations will determine the efficiency of Phl p 5b-based ATs in an established Phl p 5b-allergic mouse model. Future investigations are likely to profit from the wider availability of human Phl p 5b-reactive material.

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